Enhanced Hypothalamic AMP-Activated Protein Kinase Activity Contributes to Hyperphagia in Diabetic Rats

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AMP-activated protein kinase (AMPK) acts as a cellular energy sensor, being activated during states of low energy charge. Hypothalamic AMPK activity is altered by hormonal and metabolic signals and mediates the feeding response. To determine the effect of diabetes on hypothalamic AMPK activity, we assayed this activity in streptozotocin (STZ)-induced diabetic rats. Compared with control rats, STZ-induced diabetic rats had significant hyperphagia and weight loss. Hypothalamic AMPK phosphorylation and α2-AMPK activity were higher and acetyl-CoA carboxylase activity was lower in diabetic rats than in control rats. Chronic insulin treatment or suppression of hypothalamic AMPK activity completely prevented diabetes-induced changes in food intake as well as in hypothalamic AMPK activity and mRNA expression of neuropeptide Y and proopiomelanocortin. Plasma leptin and insulin levels were profoundly decreased in diabetic rats. Intracerebroventricular administration of leptin and insulin reduced hyperphagia and the enhanced hypothalamic AMPK activity in diabetic rats. These data suggest that leptin and insulin deficiencies in diabetes lead to increased hypothalamic AMPK activity, which contributes to the development of diabetic hyperphagia. *Diabetes* 54:63–68, 2005

Diabetes is characterized by altered fuel metabolism due to relative or absolute deficiency of insulin. Individuals with uncontrolled diabetes commonly experience hyperphagia (1), which makes glycemic control more difficult. Several mechanisms of diabetic hyperphagia have been suggested. For example, the expression of hypothalamic orexigenic neuropeptides, neuropeptides Y (NPY) and agouti-related protein (AgRP), is increased in diabetic animals, while expression of anorexigenic proopiomelanocortin (POMC) and corticotropin-releasing peptide (CRH) is decreased (2–4). Plasma levels of leptin and insulin are decreased in streptozotocin (STZ)-induced diabetic rats, and administration of insulin and leptin prevents diabetic hyperphagia and normalizes the levels of hypothalamic neuropeptides (5–8). These findings suggest that in diabetic animals, a deficiency of two hormones, insulin and leptin, causes hyperphagia by altering the balance of hypothalamic neuropeptides, but the intraneuronal signaling mechanisms causing diabetic hyperphagia are still incompletely understood.

AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that is activated when cellular energy is depleted (9,10). Once activated, the enzyme reduces the activities of ATP-consuming anabolic pathways and increases the activities of energy-producing catabolic pathways, acting to reestablish normal cellular energy balance.

AMPK is also expressed in the hypothalamic neurons involved in the regulation of food intake (11). Recent studies by our group and others have demonstrated the importance of hypothalamic AMPK in regulating food intake (12–14). Food intake and body weight were increased by overexpression of the constitutively active AMPK gene but were decreased by overexpression of the dominant-negative AMPK gene in the hypothalamus (13). Moreover, intracerebroventricular (ICV) administration of insulin or leptin decreased hypothalamic AMPK activities (13,14).

From these results, we hypothesized that deficiencies of leptin and insulin in diabetes may cause an increase in hypothalamic AMPK activities, which contributes to the development of diabetic hyperphagia. We therefore assayed changes in hypothalamic AMPK activities in STZ-induced diabetic rats and the role of hypothalamic AMPK in the development of diabetic hyperphagia.

RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats weighing 300–350 g were maintained at ambient temperature (22 ± 1°C) and with 12:12-h light-dark cycles (lights on 7:00 A.M.) with free access to water and rat diet unless otherwise indicated. All procedures were approved by the Institutional Animal Care and Use Committee at the Asan Institute for Life Sciences (Seoul, Korea).

Diabetes was induced by a single intraperitoneal injection of 65 mg/kg STZ (Sigma, St. Louis, MO), as previously described (15). Control rats received an intraperitoneal injection of an equal volume of citrate buffer. Successful induction of diabetes was defined as a blood glucose level of >11.1 mmol/l. Three days after STZ injection, the diabetic rats were randomly assigned to receive insulin treatment or no treatment (n = 10 each group). For the insulin-treated group, intermediate-acting insulin (Humulin-N; Eli Lilly, Indianapolis, IN) was administered each day just before the dark period (between 1600 and 1700) until the animals were killed. Blood glucose levels were measured each day just before insulin injection. Doses of insulin (5–7 units per

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ACC, acetyl-CoA carboxylase; AgRP, agouti-related protein; AMPK, AMP-activated protein kinase; CPT-1, carnitine palmitoyltransferase-1; CRH, corticotropin-releasing peptide; ICV, intracerebroventricular; NPY, neuropeptide Y; POMC, proopiomelanocortin; STZ, streptozotocin.

TABLE 1
Change in body weight, food intake, and plasma metabolic parameters

	Control	Diabetes	Diabetes + insulin
Changes in body weight (g)	80.0 ± 10.0	10.0 ± 5.0*	62 ± 5.2†
Epididymal fat weight (g)	5.4 ± 0.2	2.5 ± 0.2*	4.8 ± 0.2†
Average food intake (g/day)	29.5 ± 0.6	42.2 ± 1.3*	30.9 ± 0.6†
Average plasma glucose (mmol/l)	6.1 ± 0.1	26.0 ± 0.9*	10.3 ± 0.4*†
Plasma insulin (pmol/l)	500 ± 103	86 ± 68*	465 ± 120†
Plasma leptin (pmol/l)	161 ± 27	34 ± 16*	160 ± 11†

Data are means ± SE. Diabetes was induced by intraperitoneal injection of STZ, and rats were injected daily with saline or insulin for 3 weeks (n = 7–10 per group). *$P < 0.005$ vs. control group; †$P < 0.005$ vs. diabetic group.

rat) were adjusted to reach target blood glucose levels of 7.8 to 11.1 mmol/l, and food intake and body weight were monitored daily.

On the 21st day, the rats were killed by decapitation between 0900 and 1100 following an overnight fast. The medial part of the hypothalamus was dissected by the anterior border of the optic chiasm, the posterior border of the mammillary body, the upper border of the anterior commissure, and the lateral border half way from the lateral sulcus in the ventral side of brain. By microscopic examination, we confirmed that each medial hypothalamus included the arcuate nucleus, paraventricular nucleus, and ventromedial and dorsomedial nuclei. Liver and skeletal muscle tissues were obtained after freezing in situ by aluminum tongs precooled in liquid nitrogen (16). Trunk blood was obtained, and plasma was separated by centrifugation (400 g) for 15 min at 4°C. Blood and tissues were stored at −70°C until assayed.

Microdissection of the hypothalamic nuclei. Rats administered vehicle or STZ (n = 5–7) were killed 21 days later, and the whole brains were rapidly removed and frozen in isopentane on dry ice. Individual hypothalamic nuclei were dissected using a "micropunch" technique as described previously (17).

ICV cannulation and injection. Twenty-three gauge stainless steel cannulae (Plastics One, Roanoke, VA) were implanted into the third cerebral ventricle (ICV) of rats as previously described (18). Following a 7-day recovery period, the correct positioning of each cannula was confirmed by a positive dipsogenic response to ICV administration of angiotensin II (150 ng/rat). Diabetes was induced by STZ as described above. Twenty-one days later and following a 24-h fast, rats (n = 6–7) were injected intracerebroventricularly with 10 μl of vehicle (saline or DMSO), leptin (0.5 nmol), insulin (300 nmol), or the AMPK inhibitor, compound C (100 and 300 nmol; Merck, Whitehouse Station, NJ) (19) in the early light phase. Food intake was monitored for 1 h postinjection, and the rats were killed by decapitation. The medial hypothalamus was harvested for determination of AMPK activity or mRNA expression of neuropeptides.

Adenovirus-mediated gene transfer. Plasmids encoding c-*myc*-tagged forms of dominant-negative α1-AMPK with a mutation altering Asp-157 to alanine (20) and of α2-AMPK with a mutation altering Lys-45 to arginine (21) were a gift from Dr. J. Ha (Department of Molecular Biology, Kyunghee University College of Medicine, Seoul, Korea). On the 21st day after diabetes induction, rats were randomly divided into two groups (n = 9–10). Rats were injected with either 1 μl of adenoviruses expressing β-gal (10^{11} pfu/ml) or a mixture (1:1 vol) of adenoviruses expressing dominant-negative α1-AMPK and dominant-negative α2-AMPK (10^{11} pfu/ml) into the bilateral mediobasal hypothalamus as previously described (12). Injection site was confirmed by β-gal staining as previously described (22). Food intake and body weight were monitored daily. In the early light phase (0900 to 1000) of the 5th day, rats were killed by decapitation, and the medial hypothalamus was collected.

Immunoblot analysis. Immunoblot analysis was conducted as previously described (23) using antibodies directed against the phosphorylated (Thr-172) and total forms of AMPK (Cell Signaling, Beverly, MA) or phosphorylated (Ser-79) acetyl-CoA carboxylase (ACC) (Upstate Biotech, Waltham, MA). To measure total ACC protein expression, membrane was incubated with streptavidin instead of primary antibody. Band density was corrected by the density of β-actin.

AMPK activity. For measurement of isoform-specific AMPK activity, 40 μg of tissue lysates was immunoprecipitated by incubation with specific antibodies against the α1- and α2-AMPK catalytic subunits (Upstate) and 15 μl of 25% (wt/vol) protein G-sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. AMPK activity was determined using the modified method of Davies et al. (24) with these fractionated proteins in kinase assay buffer (62.5 mmol/l HEPES, pH 7.0, 62.5 mmol/l NaCl, 62.5 mmol/l NaF, 6.25 mmol/l sodium pyrophosphate, 1.25 mmol/l EDTA, 1.25 mmol/l EGTA, and 1 mmol/l dithiothreitol) containing 200 μmol/l AMP, ATP mixture (200 μmol/l ATP and 1.5 μCi of [-^{32}P]ATP), with or without 200 μmol/l SAMS peptide (HMRSAMSGLHLVKRR) at 30°C for 10 min. The reaction was terminated by spotting the reaction mixture on phosphocellulose paper (P81), and the paper was extensively washed with 150 mmol/l phosphoric acid. The radioactivity was measured with a scintillation counter.

Carnitine palmitoyltransferase-1 activity. Hypothalamic carnitine palmitoyltransferase-1 (CPT-1) activity was measured as described (25).

Semi-quantitative RT-PCR. NPY and POMC mRNA expression was quantified by semiquantitative RT-PCR using primer sets for NPY (5′-TAG GTA ACA AAC GAA TGG GG-3′ and 5′-GTC TTC AAG CCT TGT TCT GG-3′), POMC (5′-ATG CCG AGA TTC TGC TAC AG-3′ and 5′-ATG ATG GCG TTC TTG AAG AG-3′), or glyceraldehyde-3-phosphate dehydrogenase (5′-ACC ACA GTC CAT GCC ATC AC-3′ and 5′-TCC ACC ACC CTG TTG CTG TA-3′). The amplification protocol consisted of 27 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 2 min.

Measurement of blood samples. Plasma glucose was determined using a glucose analyzer (YSI 2300; Yellow Springs Instruments, Yellow Springs, OH). Plasma insulin and leptin concentrations were determined by radioimmunoassay (Linco, St. Louis, MO).

Statistical analysis. All data are presented as mean ± SE. Comparisons between groups were by unpaired Student's t test or ANOVA followed by the post hoc least significance difference test. Significance was defined as $P < 0.05$.

RESULTS

Changes in body weight, food intake, and plasma metabolic parameters in STZ-induced diabetic rats. Weight gain during the study period was significantly less in diabetic rats than in control rats (Table 1). Epididymal fat mass at the end of the study was also less in the diabetic rats (Table 1). Average food intake was greater in the diabetic rats than in control rats (Table 1). Plasma insulin and leptin levels at the end of the study period were significantly lower in the diabetic rats than in the control rats (Table 1).

Changes in AMPK activities in the hypothalamus of STZ-induced diabetic rats. Hypothalamic AMPK phosphorylation, an indicator of AMPK activation, was about two times higher in diabetic rats compared with control rats (Fig. 1*A*). Hypothalamic total AMPK protein levels tended to increase in diabetic rats, but the difference did not reach statistical significance (Fig. 1*B*). α2-AMPK activity was about 1.5-fold higher in the hypothalamus of diabetic rats, while there was no significant difference in hypothalamic α1-AMPK activity (Fig. 1*C* and *D*). α2-AMPK protein levels in the hypothalamus were not different between control and diabetic rats (data not shown).

When we analyzed AMPK activities in discrete hypothalamic nuclei, α2-AMPK activity was significantly higher in the medial part of the hypothalamus, including the arcuate

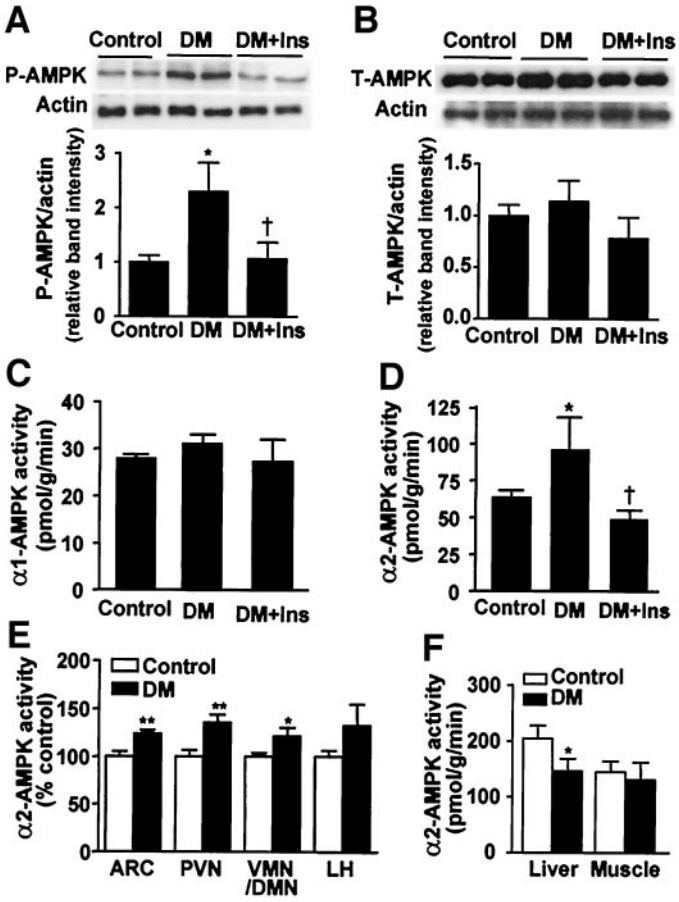

FIG. 1. Western blot analysis of phospho-AMPK (*A*), total AMPK (*B*), α1-AMPK (*C*), and α2-AMPK activities (*D*) in the hypothalamus and α2-AMPK activities in various hypothalamic nuclei (*E*) and liver and skeletal muscle (*F*) of diabetic rats (n = 7–10 per group). *$P < 0.05$ vs. control group; †$P < 0.05$ vs. diabetic group, **$P < 0.01$ vs. control.

nucleus, paraventricular nucleus, and ventromedial and dorsomedial nuclei of diabetic rats compared with similar structures in control rats (Fig. 1*E*). In contrast to the hypothalamus, α2-AMPK activities were lower in the livers of diabetic rats compared with control rats, but there were no differences in skeletal muscle α2-AMPK activities (Fig. 1*F*).

Changes in ACC and CPT-1 activities in the hypothalamus of STZ-induced diabetic rats. AMPK inhibits ACC activity through phosphorylation (9,10). In the hypothalamus of diabetic rats, ACC phosphorylation was higher (i.e., ACC activity was lower) compared with control rats (Fig. 2*A*). Hypothalamic α-ACC phosphorylation was 2.5-fold higher and hypothalamic β-ACC phosphorylation was 5.8-fold higher in diabetic rats than in control rats (Fig. 2*B* and *C*). Total ACC protein expression was not changed by induction of diabetes (Fig. 2*A*).

Reduced ACC activity has been shown to increase fat oxidation in skeletal muscle by decreasing the intracellular level of malonyl CoA, a potent inhibitor of CPT-1 (9,10). Consistent with the changes in hypothalamic ACC activity, we found that hypothalamic CPT-1 activity was higher in diabetic rats than in control rats (Fig. 2*D*).

Effects of chronic insulin treatment on hypothalamic AMPK and ACC activities in STZ-induced diabetic rats. Consistent with previous reports (6–8), chronic insulin treatment completely reversed diabetes-induced changes in body weight, fat weight, and food intake, as well as in plasma leptin and insulin levels (Table 1). In contrast, plasma glucose levels were still higher in the insulin-treated diabetic rats than in control rats.

In insulin-treated diabetic rats, hypothalamic AMPK phosphorylation decreased to the level observed in control rats (Fig. 1*A* and *B*). Although insulin treatment reduced hypothalamic α2-AMPK activity, it had no effect on α1-AMPK activity (Fig. 1*C* and *D*). Insulin treatment decreased hypothalamic α-ACC and β-ACC phosphorylation to a level below that observed in control rats (Fig. 2*A*–*C*). Increased hypothalamic CPT-1 activity in diabetic rats was also reduced by chronic treatment of insulin (Fig. 2*D*).

Chemical and molecular inhibition of hypothalamic AMPK reverses diabetes-induced changes in food intake, hypothalamic AMPK activities, and neuropeptide expression. To investigate whether enhanced hypo-

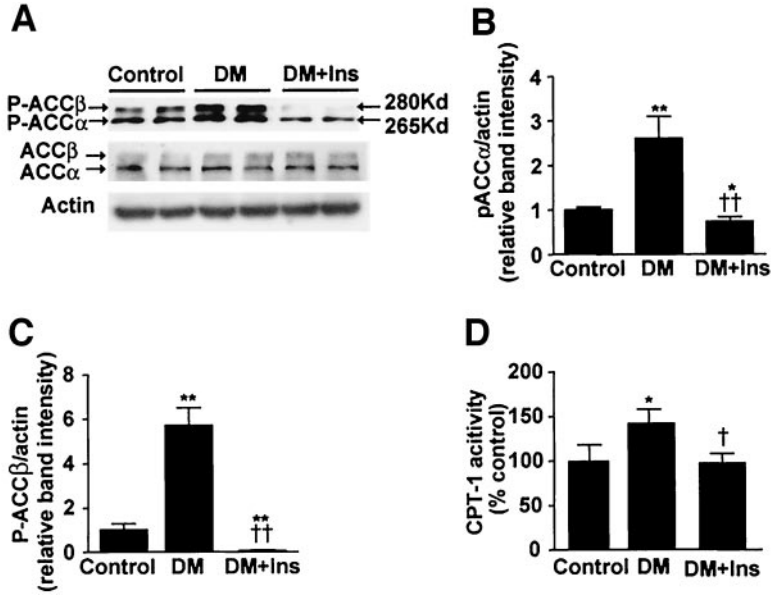

FIG. 2. Western blot analysis of phospho-ACC and total ACC (*A-C*) and CPT-1 activity (*D*) in the hypothalamus. Band intensity of phospho-ACCα and ACCβ in the hypothalamus was normalized relative to that of β-actin (n = 7–10 per group). *$P < 0.05$, **$P < 0.01$ vs. control group; †$P < 0.05$, ††$P < 0.01$ vs. diabetic rats.

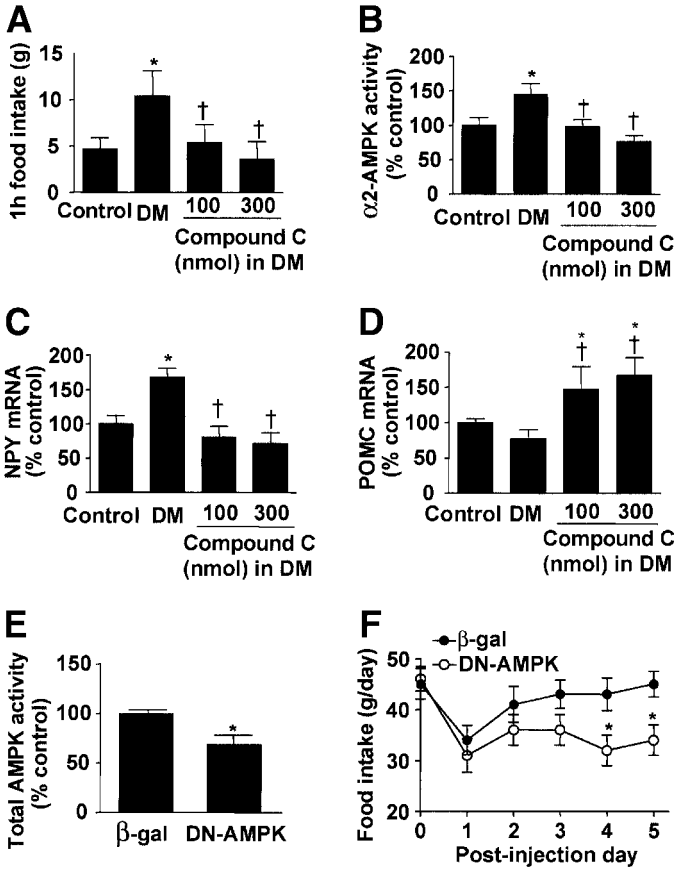

FIG. 3. Effects of ICV administration of AMPK inhibitor, compound C, on 1-h food intake (*A*), hypothalamic α2-AMPK activity (*B*), and NPY (*C*) and POMC mRNA levels (*D*) in diabetic rats. Three weeks after induction of diabetes, rats were fasted for 24 h and administered DMSO or compound C (100 and 300 nmol) (n = 8–10 per group). *P < 0.05 vs. control group; †P < 0.05 vs. vehicle-injected diabetic rats. Effects of dominant-negative AMPK overexpression in the mediobasal hypothalamus on hypothalamic α2-AMPK activity (*E*) and food intake (*F*) (n = 9–10 per group). *P < 0.05 vs. β-gal control.

thalamic AMPK activity contributes to diabetic hyperphagia, compound C, an AMPK inhibitor (19), was administered intracerebroventricularly in diabetic rats. While diabetic rats consumed larger amounts of food than control rats after a 24-h fast, ICV administration of compound C (100 and 300 nmol) reduced food intake to the level of nondiabetic control rats (Fig. 3*A*). Hypothalamic α2-AMPK activity was increased in diabetic rats and reduced by ICV administration of compound C (Fig. 3*B*). Expression of mRNA encoding the orexigenic NPY, which was higher in the hypothalamus of diabetic rats, was decreased by ICV administration of compound C (Fig. 3*C*). In contrast, anorexigenic POMC mRNA was increased by ICV compound C (Fig. 3*D*).

To ensure the effect of AMPK inhibition on diabetic hyperphagia, adenovirus-containing dominant-negative AMPK was injected into the bilateral mediobasal hypothalamus of diabetic rats. Overexpression of dominant-negative AMPK decreased total hypothalamic AMPK activity by ~30% (Fig. 3*E*). Food intake was significantly reduced in rats expressing dominant-negative AMPK in their hypothalamus (Fig. 3*F*).

Deficiency of insulin and leptin contributes to hypothalamic AMPK activation and hyperphagia in diabetes. Plasma leptin and insulin levels were significantly lower in diabetic rats compared with control rats (Table 1). It has been shown that administration of leptin or insulin reduces hypothalamic AMPK activities (13,14). To test if the deficiency of these two hormones and the resultant hypothalamic AMPK activation may contribute to diabetic hyperphagia, we administered leptin and insulin into the third ventricle of diabetic rats. ICV administration of leptin and insulin reduced food intake as well as α2-AMPK activity in diabetic rats (Fig. 4*A* and *B*). Similarly, ICV leptin and insulin reversed diabetes-induced changes in NPY and POMC mRNA expression (Fig. 4, *C* and *D*).

DISCUSSION

We have shown here that AMPK phosphorylation and α2-AMPK activities are increased in the hypothalamus of STZ-induced diabetic rats. Because food intake is increased by AMPK activation in the hypothalamus but reduced by AMPK inhibition (12–14), our results suggest that the enhanced hypothalamic AMPK activity observed in diabetic rats can cause this increase in food intake. This notion is supported by our finding that hypothalamic AMPK inhibition reduced diabetic hyperphagia.

The mechanism by which hypothalamic AMPK regulates food intake, however, remains unclear. AMPK activation is known to decrease ACC activity and intracellular levels of malonyl CoA. Malonyl CoA has been hypothesized to act as an inhibitor of food intake (26). Thus, a reduction in malonyl CoA levels could increase food intake. Alternatively, reduced malonyl CoA levels stimulate CPT-1 activity (9,10). Chemical or molecular inhibition of hypothalamic CPT-1 activity has been shown to decrease food intake (17). Thus, enhanced hypothalamic CPT-1 activity in diabetic rats

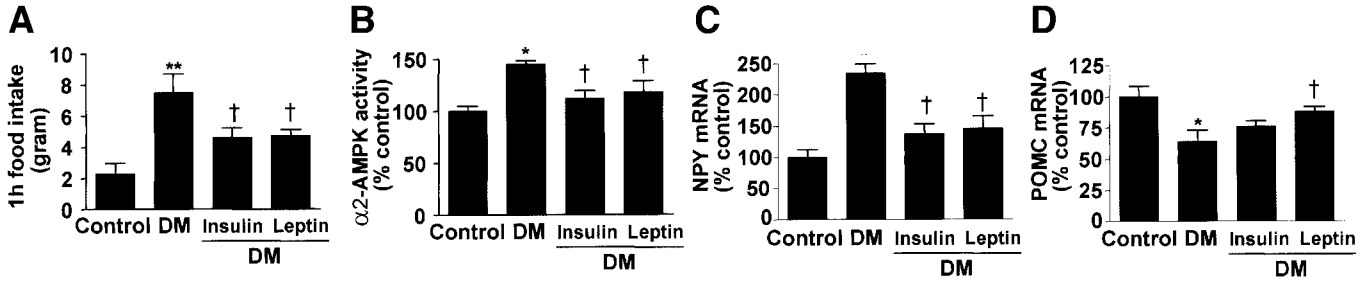

FIG. 4. Effects of ICV administration of insulin and leptin on 1-h food intake (*A*), hypothalamic α2-AMPK activity (*B*), and NPY and POMC mRNA levels (*C* and *D*) in diabetic rats. Three weeks after induction of diabetes, rats were fasted overnight and received ICV injection of saline, insulin, or leptin (n = 6–7 per group). *P < 0.05, **P < 0.01 vs. control group; †P < 0.05 vs. saline-injected diabetic rats.

may cause hyperphagia. In addition, we have shown that ICV administration of the AMPK inhibitor compound C reduced orexigenic NPY mRNA levels and increased anorexigenic POMC mRNA levels. Thus, changes in hypothalamic AMPK activity may also affect the expression of mRNA-encoding hypothalamic neuropeptides through as yet unknown mechanisms.

A number of factors may be involved in the diabetes-induced changes in hypothalamic AMPK. In our study, plasma insulin and leptin concentration was profoundly reduced in diabetic animals. Moreover, ICV administration of insulin and leptin inhibited the increase in hypothalamic AMPK activity and food intake. Thus, deficiencies of leptin and insulin may be a major contributor to the increase in hypothalamic AMPK activity observed in diabetic rats. Previous studies have shown that hypothalamic AMPK activity was decreased by glucose (12,13). Taken together, inhibition of hypothalamic AMPK by high glucose in chronic diabetic state may be insufficient to overcome AMPK activation by insulin and leptin deficiency.

Interestingly, diabetes-induced changes in AMPK activity were tissue specific. We found that AMPK activity was increased in the hypothalamus of diabetic rats but decreased in the liver and unchanged in skeletal muscle. Similarly, administration of leptin (27) and α–lipoic acid (12) (K.H. Song, J.Y.P., J.M. Koh, H.S. Kim, H.S.P., H.J. Park, M.S.K., J. Ha, J.H. Youn, K.U.L., unpublished data) stimulated AMPK activity in skeletal muscle but suppressed this activity in the hypothalamus. Additional studies are needed to clarify the molecular mechanism by which similar metabolic conditions or agents cause differential changes in AMPK activity in different tissues.

In summary, we have shown here that hypothalamic AMPK phosphorylation and activity were increased in diabetic rats. Inhibition of hypothalamic AMPK activity decreased food intake and normalized hypothalamic neuropeptide expression in diabetic rats. These results demonstrate that increased hypothalamic AMPK activity contributes to the development of diabetic hyperphagia.

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